The Role of Hydrophobic Side Chains as Determinants of Antibacterial Activity of Semisynthetic Glycopeptide Antibiotics

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Vancomycin, LY264826 and four *N*-substituted derivatives of LY264826 were examined for dimerization, binding to D-alanyl-D-alanine- and D-alanyl-D-lactate-containing cell wall ligands, and binding to bacterial membrane vesicles. The six glycopeptide antibiotics represent a 360-fold range in antibacterial activities against *Micrococcus luteus* (MIC = 0.00072 ~ 0.26 μM) with the *N*-substituted compounds having the lowest MICs. Vancomycin, LY264826 and the four *N*-substituted derivatives shared nearly identical binding affinities for *N*,*N*'-diacetyl-L-lysyl-D-alanyl-D-alanine ($K_b = 1.5 \times 10^5 \sim 5.9 \times 10^5$ M^{-1}). Affinities for binding *N*,*N*'-diacetyl-L-lysyl-D-alanyl-D-lactate were lower but also represented a narrow range ($K_b = 0.24 \times 10^3 \sim 1.6 \times 10^3$ M^{-1}). In contrast to ligand binding, the relative capacity of the six compounds to dimerize differed by four orders of magnitude ($K_{dim} = 4.9 \times 10^1 \sim 1.2 \times 10^6$ M^{-1}). The *N*-substituted derivatives had the highest K_{dim} values, required the greatest molar excess of exogenous cell wall ligand to suppress inhibition, and demonstrated a propensity to bind to bacterial membrane vesicles. The derivatives with the most lipophilic side chains were the most highly bound to vesicles. The findings suggest that the enhanced antibacterial activities of *N*-substituted derivatives of LY264826 derive from the nature of the hydrophobic side chain which can have a marked effect on dimerization and membrane binding.

The glycopeptide antibiotics consist of a rigid, linear heptapeptide backbone which may be substituted with a variety of amino and nonamino sugars. The amino sugar moieties of some members of this class contain *N*-acyl, *N*-alkyl, or *N*-aryl substitutions[1,2]. As a group, the glycopeptides inhibit bacterial cell wall biosynthesis by binding to key peptidoglycan intermediates[3~6]. The heptapeptide portion of these agents forms a carboxylate binding pocket which can bind the D-alanyl-D-alanine (D-Ala-D-Ala) peptidyl terminus of the disaccharide pentapeptide intermediate. A combination of five hydrogen bonds plus favorable hydrophobic effects facilitates highly specific binding and leads to inhibition of the transglycosylation reaction in glycopeptide-susceptible bacteria[5~7]. Vancomycin is the most commonly cited member of the glycopeptide class as it is extensively used to treat serious infections caused by Gram-positive bacteria.

LY264826 (A82846B) is a glycopeptide antibiotic with a structure similar to vancomycin but with an additional amino sugar (4-*epi*-vancosamine) attached at residue 6 of the linear heptapeptide and contains 4-*epi*-vancosamine substituted for vancosamine in the disaccharide attached at residue 4 (Fig. 1). The antibacterial activity of LY264826 is, on average, four to eight fold greater than that of vancomycin against several Gram-positive species[8~10]. Despite differences in activities, LY264826 and vancomycin share nearly identical affinities for binding the D-Ala-D-Ala-containing tripeptide ligand, *N*,*N*'-diacetyl-L-lysyl-D-alanyl-D-alanine (*N*,*N*'-diacetyl-L-Lys-D-Ala-D-Ala)[11,12]. MM 45289 (also referred to as eremomycin or A82846A) is closely related to LY264826[13] and has enhanced activity over vancomycin. In a study by GOOD, *et al.*[14] the affinity of MM 45289 for binding *N*,*N*'-diacetyl-L-Lys-D-Ala-D-Ala was reported to be 23-fold less than the affinity of vancomycin for the same ligand. The authors concluded that their *in vitro* binding experiments did not serve as a useful model of the glycopeptide peptidoglycan target.

Vancomycin and other glycopeptides can self-associate to form homodimers[15~17]. Vancomycin is relatively poor at dimer formation[12,16,17]; by comparison, glycopeptides such as LY264826 and eremomycin have a much greater capacity to dimerize[11,12,17,18]. Based on results from NMR and microbiological studies[12,17,19~21], WILLIAMS and coworkers have proposed that dimerization plays an important role in determining antibacterial activity. The studies from this group showed that affinity of certain glycopeptide antibiotics for cell wall analogs was enhanced by dimerization, and that dimerization, in

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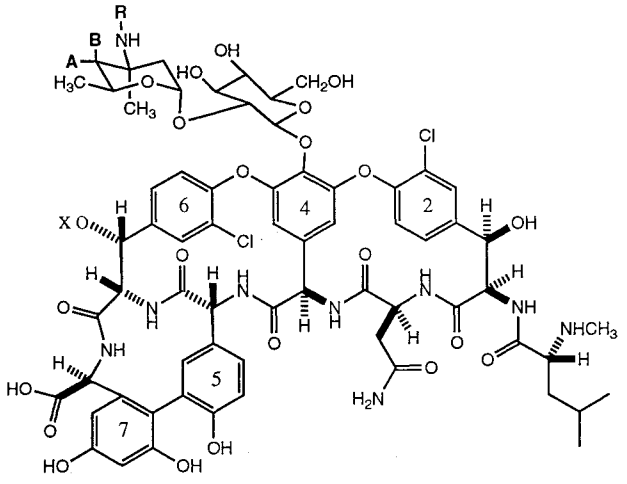
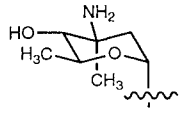
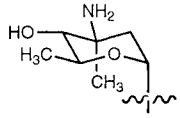
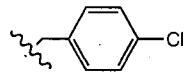
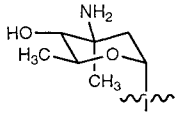
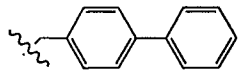
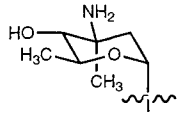
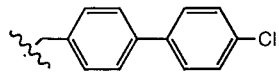
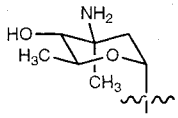
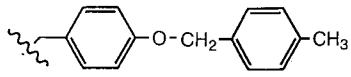
Fig. 1. Chemical structures of glycopeptide antibiotics examined in this study.



	A	B	X	R
Vancomycin	H	OH	H	H
LY264826	OH	H		H
LY191145	OH	H		
LY307599	OH	H		
LY333328	OH	H		
LY377502	OH	H		

turn, was enhanced by the presence of the cell wall analogs. These findings revealed a subtle balance between dimerization and ligand binding in determining the antibacterial activity of these agents[12].

Semisynthetic *N*-substituted derivatives of LY264826 (comprising both alkyl and aryl side chain substituents) having enhanced antibacterial activity against both vancomycin-susceptible and -resistant bacteria have been described[1,9,22,23]. LY191145 is a 4-chlorobenzyl derivative of LY264826. A recent study[11] using a capillary electrophoretic method showed that this *N*-substituted compound dimerized as well or better than the parent compound lacking a side chain. Moreover, the same study revealed that the hydrophobic side chain of LY191145 facilitated interaction with bacterial membranes. In the present report, we have compared four *N*-substituted derivatives of LY264826 which differ only in composition of the side chain but represent a 30-fold range in antibacterial potency. The findings indicate that the nature of the hydrophobic side chain influences the extent of dimerization and membrane binding.

Materials and Methods

Chemicals

The natural glycopeptides (vancomycin and LY264826 [A82846B]) and semisynthetic *N*-substituted derivatives

of LY264826 (LY191145 [4-chlorobenzyl], LY307599 [4-phenylbenzyl], LY333328 [4-(4′-chlorophenyl)benzyl], and LY377502 [4-(4′-methylbenzyloxy)benzyl]) were from Eli Lilly and Company. *N,N′*-Diacetyl-L-Lys-D-Ala-D-Ala was purchased from Sigma Chemical Co. *N,N′*-Diacetyl-L-Lys-D-Ala-D-Lac was purchased from Bachem. Biochem. Inc.

Ligand Binding

Binding of *N,N′*-diacetyl-L-Lys-D-Ala-D-Ala and *N,N′*-diacetyl-L-Lys-D-Ala-D-Lac to glycopeptides was measured by affinity capillary electrophoresis[11,24] using a Beckman P/ACE 5000 fitted with a UV detector (214 nm) and a 57 cm × 75 μm i.d. uncoated fused silica capillary. Glycopeptides were dissolved in 10~20 mM sodium phosphate buffer (pH 7.1) and injected at 0.5 psi for 4 sec. Tripeptide and tridepsipeptide ligands were included in the run buffer (20~75 mM sodium phosphate buffer [pH 7.1]; ionic strength was varied as explained previously[11]). Mesityl oxide was used as neutral marker. The change in mobility of the glycopeptide ($\Delta\mu_G$) as a function of ligand concentration [L] (corrected for contributions of electroosmotic flow independent of [L][25]) was determined as

$$\Delta\mu_{G,L} = l_c l_d / V[(1/t_{G,L} - 1/t_{EO,L}) - (1/t_G - 1/t_{EO})]$$

where l_c is the overall capillary length, l_d is the length of the capillary from inlet to detector, V is voltage, t_G and t_{EO} are the migration times of the glycopeptide and neutral marker, respectively, and $t_{G,L}$ and $t_{EO,L}$ are the migration times of the glycopeptide and neutral marker at ligand concentration [L], respectively. Values of $\Delta\mu_{G,L}$ measured at various [L] were analyzed by Scatchard analysis according to the following relationship

$$\Delta\mu_{G,L}/[L] = K_b\Delta\mu_{G,L}^{max} - K_b\Delta\mu_{G,L}$$

where K_b and $\Delta\mu_{G,L}^{max}$, respectively, represent the binding constant and mobility of glycopeptide when saturated with ligand[24,25].

Dimerization

Dimerization of glycopeptide antibiotics was measured by capillary zone electrophoresis as previously described[11,26]. Electrophoresis equipment, buffers and conditions were the same as described for measuring ligand binding. Dimerization constants (K_{dim}) were calculated from the measured mobility (μ_{obs}) and the relationship:

$$\mu_{obs} = \frac{2\mu_m - \mu_d + \mu_d\sqrt{1+8K_{dim}[G]_t}}{1+\sqrt{1+8K_{dim}[G]_t}}$$

where μ_d is the mobility of a sample containing only dimer, μ_m is the mobility of a sample containing only monomer and $[G]_t$ is the total concentration of glycopeptide (monomer + dimer). Values for μ_{obs} and $[G]_t$ were fitted to this equation by nonlinear regression analysis using a curve fitting program (JMP; SAS Institute) to estimate μ_m and μ_d and solve for K_{dim}.

Membrane Binding

Membrane vesicles were prepared from *Bacillus megaterium* by treatment with *N*-acetylmuramidase, DNAse and RNAse according to a published procedure[27]. Binding of glycopeptide antibiotics to vesicles was measured as previously described[11].

Antibacterial Activity and Antagonism Assays

MICs were determined against *Micrococcus luteus* ATCC 9341 grown in TY broth (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl) in a microplate format. Each well contained 200 μl TY broth; inocula represented a 1:40,000 dilution of an overnight culture; plates were incubated overnight at 35°C and read at 650 nm in a ThermoMax (Molecular Devices) plate reader. Antagonism of antibacterial activity by *N,N′*-diacetyl-L-Lys-D-Ala-D-Ala and *N,N′*-diacetyl-L-Lys-D-Ala-D-Lac was determined using the same microplate format and procedure except that tripeptide or tridepsipeptide ligands were added to wells prior to inoculation. Each glycopeptide concentration was tested for inhibition in the presence of a range of ligand concentrations. Molar excess (ME) is the ratio of [ligand]/[antibiotic] in the well containing the lowest ligand concentration that completely suppressed inhibition. The values given in Table 1 represent a mean ME calculated for several inhibitory concentrations of glycopeptide.

Table 1. Antibacterial activity and dimerization of glycopeptide antibiotics.

Glycopeptide[a]	MIC[b]	ME[c]	K_{dim}[d]
Vancomycin	0.26	27	$4.9 (\pm 0.16) \times 10^1$
LY264826	0.057	302	$8.6 (\pm 1.8) \times 10^3$
LY191145	0.022	1,978	$1.3 (\pm 0.4) \times 10^4$
LY307599	0.0084	16,500	$3.2 (\pm 0.4) \times 10^5$
LY333328	0.0011	284,000	$6.3 (\pm 1.0) \times 10^5$
LY377502	0.00072	262,000	$1.2 (\pm 0.5) \times 10^6$

[a] Chemical structures shown in Fig. 1.
[b] Minimal inhibitory concentration (μM) to inhibit growth of *Micrococcus luteus* ATCC 9341.
[c] Molar excess of *N,N′*-diacetyl-L-Lys-D-Ala-D-Ala ligand required to completely suppress growth inhibition of *Micrococcus luteus* ATCC 9341.
[d] Dimerization constant (K_{dim}; M^{-1}). Estimates are shown ± approximate standard error.

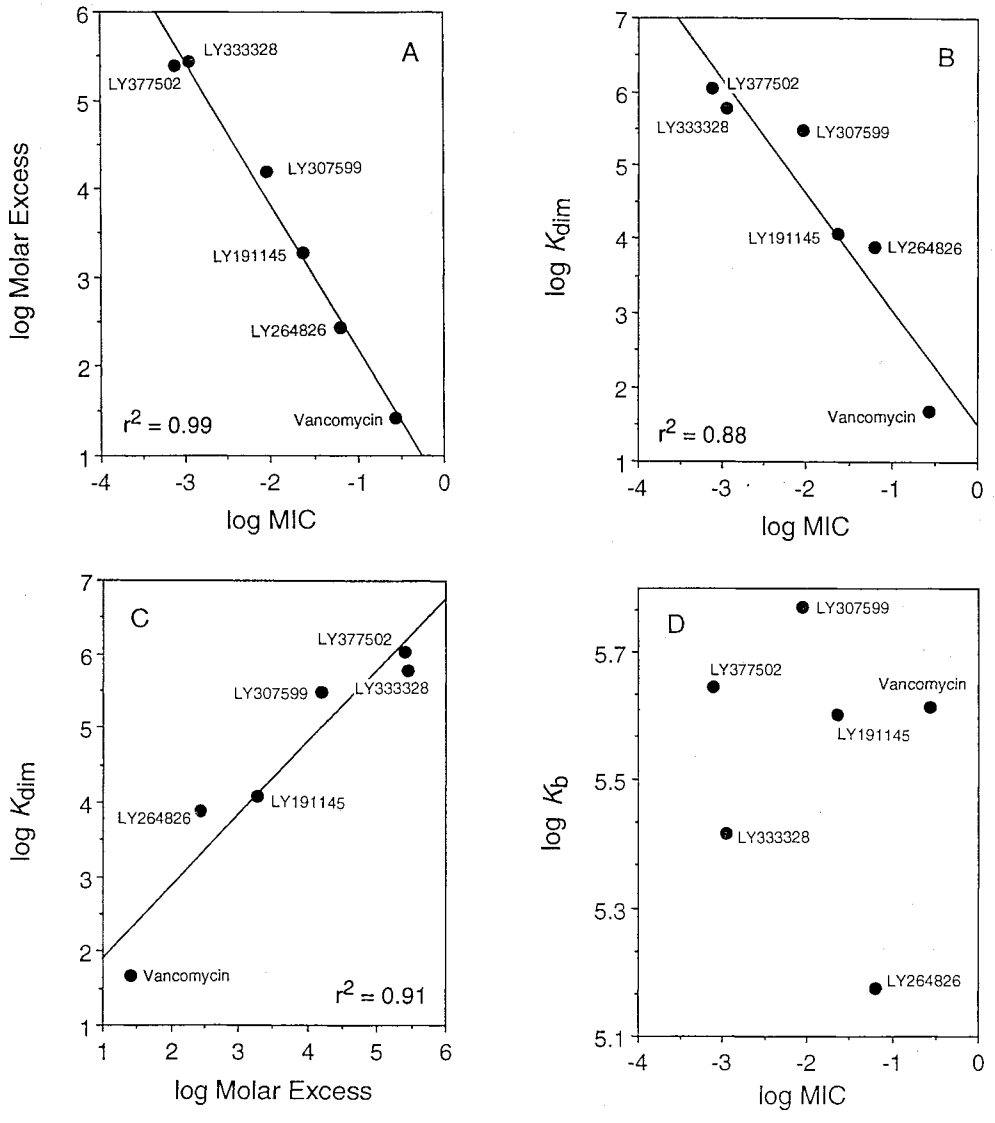
Results

Antibacterial Activity

MICs for each of the compounds tested against *M. luteus* are given in Table 1. LY264826 was at least 4-fold more active and the *N*-substituted derivatives were 12 to 360-fold more active than vancomycin. Antibacterial activities of the *N*-substituted derivatives differed by a factor of 30. Since the derivatives differ only in side chain composition[23] the 30-fold range in MIC values derives from the nature of the *N*-alkyl or *N*-aryl substitutions. Inhibition by all six agents was antagonized by the addition of excess tripeptide ligand, *N,N'*-diacetyl-L-Lys-D-Ala-D-Ala. The molar excess of ligand needed to completely suppress antibacterial activity varied from a low of 27 fold for vancomycin to 284,000 fold for LY333328. The *N*-substituted derivatives had the lowest MICs and required the greatest excess of free ligand to suppress activity. A strong inverse correlation ($r^2 = 0.99$) between ME and MIC was observed (Fig. 2A).

Other studies[9,22,28] have demonstrated that *N*-substituted derivatives of LY264826 are highly active against vancomycin-resistant enterococci. MICs for the four *N*-substituted derivatives in Table 1 against *Enterococcus faecium* 180 (vanA-type vancomycin-resistant[29]) ranged from 0.002 to 0.2 μM. Moreover, the inhibitory activity of these derivatives against *E. faecium* 180 was antagonized by cell wall ligand; ME values ranged from 180 to 68,000.

Fig. 2. Relationship between (A) molar excess of *N,N'*-diacetyl-L-Lys-D-Ala-D-Ala required to suppress inhibition and antibacterial activity (MIC); (B) dimerization and MIC; (C) dimerization and molar excess of *N,N'*-diacetyl-L-Lys-D-Ala-D-Ala required to suppress inhibition; (D) ligand binding (*N,N'*-diacetyl-L-Lys-D-Ala-D-Ala) and MIC.



Data taken from Table 1. Lines shown represent best fit from linear regression analysis. r^2 = coefficient of determination.

Table 2. Binding of cell wall ligands by glycopeptide antibiotics.

Glycopeptide[a]	K_b (D-Ala-D-Ala)[b]	K_b(D-Ala-D-Lac)[c]
Vancomycin	4.1×10^5	0.41×10^3
LY264826	1.5×10^5	1.6×10^3
LY191145	4.0×10^5	1.4×10^3
LY307599	5.9×10^5	0.35×10^3
LY333328	2.6×10^5	0.24×10^3
LY377502	4.4×10^5	0.37×10^3

[a] Chemical structures shown in Fig. 1.
[b] Binding constant (K_b; M^{-1}) for *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Ala.
[c] Binding constant (K_b; M^{-1}) for *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Lac.

Dimerization

As reported previously[11,12,17], dimerization of LY264826 is at least 100 times stronger than dimerization of vancomycin. Dimerization constants (K_{dim}) for the six agents examined in the present study were determined by a capillary electrophoretic method[26] and are presented in Table 1. K_{dim} values differed by as much as four orders of magnitude indicating that *N*-alkylation of the 4-*epi*-vancosamine on residue 4 of LY264826 has a marked effect on dimerization. The K_{dim} for the *N*-substituted derivatives of LY264826 differed by as much as 85-fold and each exceeded the K_{dim} of the parent compound.

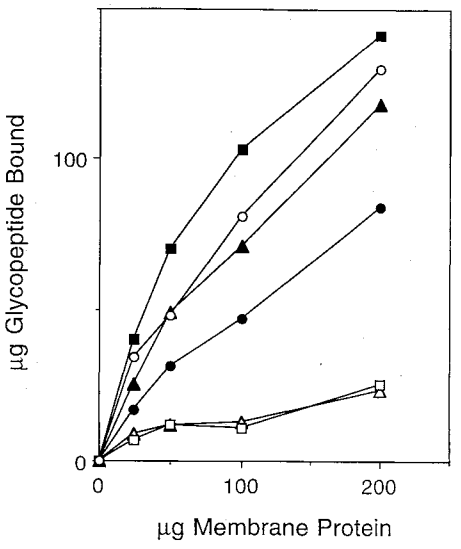
Dimerization correlated inversely with MIC (Fig. 2B; $r^2 = 0.88$) and directly with ME (Fig. 2C; $r^2 = 0.91$). The most strongly dimerized compound was the 4-(4′-methylbenzyl-oxy)benzyl derivative (LY377502) having a K_{dim} 128-fold greater than that of LY264826 and 22,000-fold greater than that of vancomycin. In addition, LY377502 had the lowest MIC against *M. luteus* and required one of the greatest excesses of free ligand (ME) to completely antagonize inhibition.

Ligand Binding Affinity

The relative binding affinities for cell wall ligands was assessed by measuring binding to *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Ala and *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Lac. Binding constants (K_b) are presented in Table 2. Despite the 360-fold range of MIC values in Table 1, the K_b values for *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Ala differed by no more than four fold indicating that binding of this ligand into the carboxylate binding pocket of each of these compounds occurs with near equal facility. There was no correlation between MIC and binding to *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Ala (Fig. 2D).

Fig. 3. Glycopeptide binding to membrane vesicles.

△, Vancomycin; □, LY264826; ●, LY191145; ▲, LY307599; ○, LY377502; ■, LY333328.



These agents also did not differ greatly with respect to their affinities for binding the lactate-containing tridepsipeptide, *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Lac. For each compound the K_b value for binding the tridepsipeptide was 100 to 1000-fold less than the K_b for the D-Ala-D-Ala-containing ligand; however, similar to binding the D-Ala-D-Ala-containing ligand, differences between individual compounds was less than seven fold (Table 2). There was very poor correlation between MIC and binding affinity for *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Lac ($r^2 = 0.26$). The antibacterial activity of LY333328 against vancomycin-resistant *E. faecium* 180 (MIC = 0.0025 μM) was 25,000-fold greater than that of vancomycin (MIC = 63 μM) yet the K_b values for these two agents differed only by a factor of two.

The differences in MICs for the *N*-substituted derivatives cannot be accounted for by the differences in binding affinity for D-Ala-D-Ala or D-Ala-D-Lac residues. The magnitude and range of both MIC and ME values indicate that the mechanism of action of these agents is not due to simple bimolecular interactions with cell wall ligands at the target site.

Membrane Binding

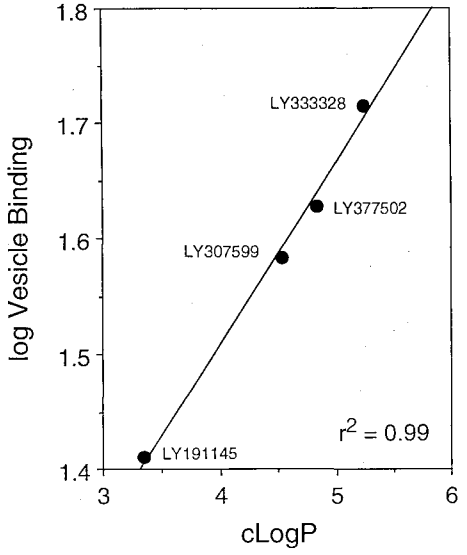
The experiment shown in Fig. 3 examined binding of the *N*-substituted derivatives of LY264826 to bacterial membrane vesicles. Neither vancomycin nor LY264826 demonstrated significant binding to these membranes[11]; however, *N*-substitution of LY264826 with an alkyl or

Table 3. Side chain lipophilicities.

Glycopeptide[a]	Lipophilicity index (cLogP)[b]
LY191145	3.354
LY307599	4.529
LY377502	4.827
LY333328	5.242

[a] Chemical structures shown in Fig. 1.
[b] cLogP values calculated for side chains as 1-octanol/water partition coefficients using the CLOGP program (D. WEININGER; Daylight Chemical Information Systems, Irvine, CA).

Fig. 4. Relationship between vesicle binding by *N*-substituted glycopeptides and cLogP values for the side chains.



Vesicle binding measurements calculated using data in Fig. 3 as nmoles of antibiotic bound to 100 μg of membrane protein. Line is best fit by linear regression analysis. r^2 = coefficient of determination.

aryl side chain conferred membrane binding activity. The *N*-substituted derivatives also were able to bind to artificial liposomes and lyse bacterial protoplasts (unpublished observations). LY377502 and LY333328 exhibited the strongest membrane interactions and were also the most strongly dimerized. Dimerization alone, however, is not sufficient for vesicle binding since LY264826 is dimerized much more strongly than vancomycin, yet neither agent showed significant binding to membrane vesicles. cLogP values for the four side chain substitutions (Table 3) indicate that vesicle binding is influenced by hydrophobicity. The agents with the most lipophilic side chains bound most strongly to vesicles; there was significant correlation ($r^2 = 0.99$) between cLogP values and vesicle binding (Fig. 4).

Discussion

Allowing that inhibition of growth of *M. luteus* by a glycopeptide antibiotic results from binding D-Ala-D-Ala termini of disaccharide pentapeptide intermediates[5] MIC measurements reflect the relative effectiveness of this interaction at the bacterial target site. But MIC values are influenced by processes that directly and indirectly affect this interaction including delivery of the glycopeptide to its target site; MIC values provide no qualitative information about the kinds of interactions that can occur at the target site to influence binding of cell wall substrates. Several studies[11,12,19,21] have shown that many glycopeptide antibiotics can form homodimers and that this can have a positive effect on antibacterial activity. Other studies[11,19,21,30] have shown that antibacterial activity of glycopeptide antibiotics may be enhanced by *N*-substitution with acyl, aryl or alkyl side chains which facilitate membrane anchoring. WILLIAMS and coworkers have made persuasive arguments that both dimerization and membrane anchoring can enhance antibacterial activity by facilitating intramolecular interactions with cell wall substrates at the target site[12,19,20].

Vancomycin-resistant enterococci (vanA and vanB types) utilize cell wall intermediates terminating in D-Ala-D-Lac in place of D-Ala-D-Ala[31~33]. The binding affinity of vancomycin for D-Ala-D-Lac residues is about 1000-fold less than the affinity for D-Ala-D-Ala which accounts for the high MICs for vancomycin (and other glycopeptides) against these organisms[11,34,35]. Semisynthetic derivatives of LY264826 containing *N*-substituted alkyl or aryl side chains demonstrate good activity against vancomycin-resistant enterococci[9,22]. Previous studies[11,28] demonstrated that the antibacterial activity of the 4-chlorobenzyl derivative (LY191145) was dependent on interaction with D-Ala-D-Ala and D-Ala-D-Lac residues leading to inhibition of the transglycosylation reaction. The studies provided evidence that the effectiveness of LY191145 resulted from intramolecular interactions with cell wall substrates facilitated by a combination of strong dimerization and membrane anchoring.

The comparison of glycopeptide antibiotics in the present study shows that *N*-substitution of LY264826 with alkyl or aryl side chains enhances antibacterial acitivity and that the nature of the side chain influences the magnitude of the increase. MICs for all six agents

correlated inversely with the extent of dimerization as measured directly by capillary electrophoresis (K_{dim} values) and indirectly by ligand antagonism experiments (ME values); note that there was a direct correlation between K_{dim} and ME values (Fig. 2C). Moreover, the *N*-substituted derivatives with the lowest MICs were the most highly bound to membrane vesicles; these derivatives also had the most hydrophobic sidechains. However, all six agents shared similar affinities for binding cell wall ligands (both D-Ala-D-Ala- and D-Ala-D-Lac-containing) indicating that although binding cell wall substrates by glycopeptides is essential for activity, differences in binding cannot account for the differences in MICs observed here.

Dimerization of glycopeptide antibiotics can be influenced by the structure and arrangement of sugars on the peptide backbone, and by presence of chlorine atoms on the aromatic rings. For example, dimerization of LY264826 is enhanced by the presence of a ring 6 sugar, favorable interactions between ring 4 disaccharides and the presence of a chlorine atom on ring 2[12,17]. The results reported in the present study indicate that *N*-substitution on the disaccharide of LY264826 further enhances dimerization and confers hydrophobic properties to the molecule which enable binding to membrane vesicles. Dimerization of the 4-phenylbenzyl-*N*-substituted derivative of vancomycin (the vancomycin analog corresponding to LY307599; $K_{dim}=8\times10^3\,M^{-1}$, measured by capillary electrophoresis) is two orders of magnitude greater than dimerization of unsubstituted vancomycin, and, unlike vancomycin, the derivative shows significant binding to membrane vesicles (unpublished results). As demonstrated with teicoplanin[19,21,36] side chain-facilitated membrane interactions alone can enhance antibacterial activity *via* intramolecular interactions with cell wall substrates at the bacterial surface. Classic chelate phenomena[37] predict that *N*-substituted derivatives of LY264826 and other highly dimerized glycopeptides should be even more strongly asssociated with the membrane. The pronounced effect that the hydrophobic side chains have on the antibacterial activity of the glycopeptides examined in this study likely derives from the combined effects of enhanced dimerization and membrane anchoring. The magnitude of these combined effects is sufficient to enable the clinically relevant activity of LY333328 against vancomycin-resistant enterococci[22,38].

Acknowledgments

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